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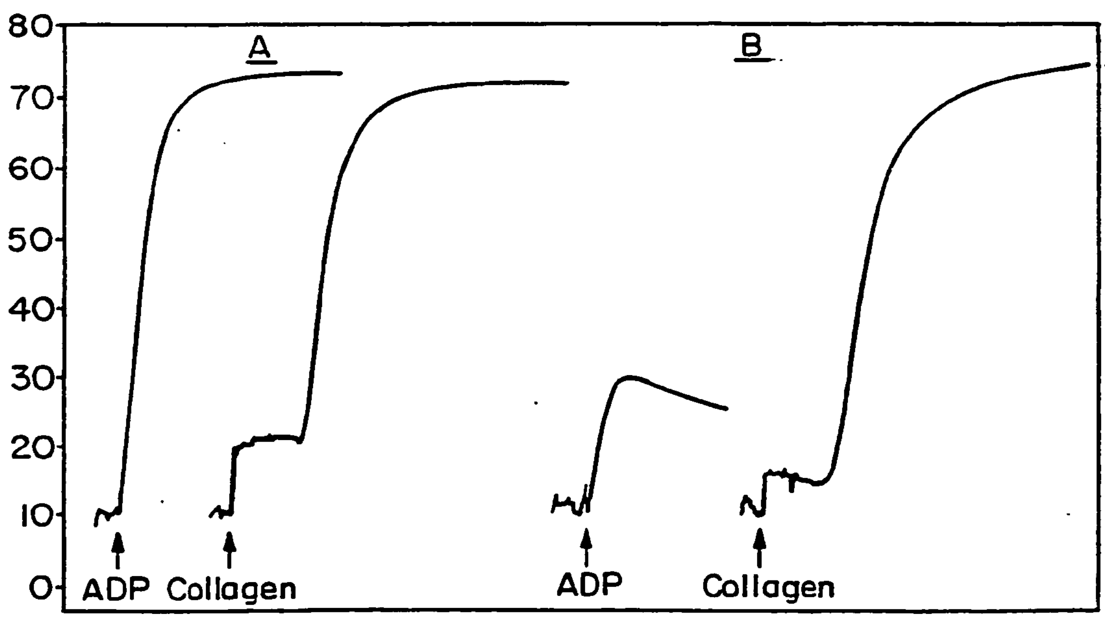

(57) Abstract

A component of blood platelets and analogues thereof are described. The invention is based on the discovery that this component, a dinucleotide, as well as several of its chemically synthesized analogues, is an effective anti-platelet and anti-thrombotic agent.

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DIADENOSINE 5', 5'''-P^1, P^4-TETRAPHOSPHATE AND ANALOGS THEREOF AS ANTITHROMBOTIC AGENTS

Description

Background of the Invention

Intravascular clotting is a common disorder. One of the most common of such disorders is the formation of thrombi, or clots, which form in a blood vessel or heart cavity and remain at the point of formation. Thrombi can have serious adverse effects on an individual. For example, thrombus formation in the heart can restrict blood flow, resulting in myocardial infarction (death of the heart muscle), which is one of the most severe forms of heart attacks.

In addition to having adverse effects at the point at which it forms, all or part of a thrombus can dislodge from its point of attachment and move through blood vessels, until it reaches a point where passage is restricted and it can no longer move. The sudden blockage of blood flow which results is referred to as a thromboembolism. The lungs are particularly susceptible to emboli

formation because it is in the lungs where main arteries first divide into smaller arteries and capillaries after the heart has received blood flow from the venous system. Emboli trapped in the lungs interfere with gas exchange and circulation. Accordingly, methods which prevent thrombi formation are of great medical importance.

Although the process of thrombus formation is only incompletely understood, two major stages have been identified: the aggregation of platelets at the site of a blood vessel injury, and the formation of a cross-linked fibrin polymer which binds the developing clot together.

The dinucleotide, diadenosine 5', 5'''-p^1, p^4-tetraphosphate (AP_4A) (Formula I), an ubiquitous component of living cells, is stored in high concentrations in the dense granules of blood platelets Zamecnik, P. C. and Stephenson, M.L., Regulatory mechanisms for protein synthesis. In: Mammalian Cells, San Pietro, A., Lamborg, M. R. and Kenney, P. C. (eds.), Academic Press, New York, pp. 3-16 (1968). AP_4A is present in normal human platelets in a concentration higher than that present in any other cellular compartment. Flodgaard, M. and Klenow, M. Biochemical Journal, 208:737-742 (1983). The stored AP_4A was thought to be metabolically inert because incubation of platelets with ^3H-adenosine results in labeled ATP but not labeled AP_4A. Thrombin treatment of platelets induces the complete release of AP_4A, along with other storage pool nucleotides, including ADP and the dinucleo-

tide, diadenosine 5', 5'''-p[1], p[3]-triphosphate (AP_3A). Luthje, J. and Ogilvie, A. Biochem. Biophys. Res. Comm. 115:253-260 (1983). AP_3A is hydrolysed in plasma to AMP (adenosine monophosphate) and ADP (adenosine diphosphate); AP_4A is degraded to AMP and ATP (adenosine triphosphate) Luthje, J. and Ogilvie, A. European Journal of Biochemistry, 149:119-127 (1985).

The precise physiological role of AP_4A has not been defined, but it has been associated with a variety of cellular metabolic events. Zamecnik, P. Anals of Biochemistry, 134:1-10 (1983). The unusually high concentration of AP_4A in platelets has led to speculation that it has a role in platelet physiology. Platelets stimulated to undergo aggregation show a second phase of aggregation upon the release of endogenous ADP stored in the dense granules. *In vitro* experiments have demonstrated that AP_4A competitively inhibits ADP-induced platelet aggregation, causing an immediate dispersion of aggregated platelets, even when aggregation has progressed to 60% completion Chao, F. C. and Zamecnik, P., Hoppe Seyler's Z. Physiol. Chem., 365:610 (1984). By contrast, AP_3A causes a gradual aggregation of platelets, most likely through its degradation product, ADP. The aggregating activity of AP_3A is immediately reversible upon the addition of AP_4A. Luthje, J. and Ogilvie, A. Biochem. Biophys. Res. Comm., 118:704-709 (1984).

Summary of the Invention

This invention is based on the discovery that administration of the dinucleotide AP_4A or an analogue thereof results in inhibition of platelet aggregation and reduction in thrombus formation. This invention relates to AP_4A and analogues of AP_4A, such as a B-B-monochloro methylene derivative, E_{10}, and their use as anti-platelet, antithrombotic agent in, for example, the prevention of coronary and cerebrovascular thromboembolic events, and in the prevention of thrombosis in hemodialysis arteriovenous shunts.

The present invention relates to a method for the prevention of thrombi formation which relies on the inhibition of platelet aggregation. It further relates to an antithrombotic agent, AP_4A, which is isolated from substances normally found in the blood in order to minimize allergic reactions, and to AP_4A analogues.

Brief Description of the Drawings

Figure 1 is a graph showing the effect of AP_4A on platelet aggregation induced by 2×10^{-5}M ADP when AP_4A is added at the midpoint of the ADP-induced secondary wave aggregation.

Figure 2 is a graph showing the effect of AP_4A (Panel A, 1×10^{-3}M, Panel B, 2×10^{-3}M) on platelet aggregation induced by collagen (200 ug/ml) when AP_4A is added at the peak of collagen-induced aggregation.

Figure 3 shows the effect of AP_4A on platelet aggregation. Panel A is a graph showing the effect of AP_4A on platelet aggregation induced by 2×10^{-5}M

ADP when the AP_4A is added before the ADP. Panel B is a graph showing the effect of AP_4A on platelet aggregation induced by collagen (200 ug/ml), when the AP_4A is added before the collagen.

Figure 4 is a graph showing the aggregation of platelets recovered from control (Panel a) and AP_4A-treated (Panel b) rabbits induced by ADP (2×10^{-5}M) and collagen (200 ug/ml).

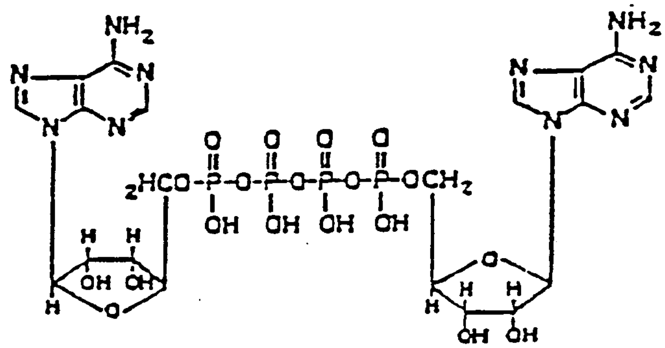
Figure 5 is a graphic representation of ADP-induced aggregation of platelets in the presence of various inhibitor analogues of AP_4A.

Figure 6 is a double-reciprocal plot showing the inhibitory effect of AP_4A and E_{10} upon ADP-induced platelet aggregation.

Detailed Description of the Invention

The subject invention relates to the use of diadenosine 5',5'''-p^1,p^4-tetraphosphate (AP_4A), or an analogue thereof, as an antithrombotic agent. The invention is based on the discovery that the administration of AP_4A, a dinucleotide present in high concentrations in the dense granules of blood platelets, or an analogue thereof, to a mammal inhibits platelet aggregation, and, therefore, reduces the incidence of thrombosis.

AP_4A has the following formula:



FORMULA I

It is also possible to apply this information to the design of antithrombotic drugs; that is, AP_4A (also represented by ApppppA) can be used as a model to design similar or more efficacious agents (e.g., synthetic analogs) to be used in the prevention of blood clots. An analog is a substance that resembles another in structure. An analog of AP_4A may have a modification in one or more of the rings of AP_4A, in one or more of substituents of AP_4A, such as an internucleotide phosphate, or in both. Examples of AP_4A analogs include App(CHCl)ppA, App(CHF)ppA, App(CH_2)ppA, App(CHBr)ppA, Appp(CH_2)pA, Ap(CH_2)pp(CH_2)pA, (Sp,Sp)$Ap_spCH_2pp_sA$, (Rp,Rp)$Ap_spCH_2pp_sA$, (Rp,Sp)$Ap_spCH_2pp_sA$ and additional analogs described by Blackburn <u>et al</u>. in <u>Nucleic Acid Research</u> <u>15</u>: 6991, 1987, the teachings of which are incorporated herein by reference. Applicants have demonstrated that the B-B'-monochloromethylene derivative of AP_4A (designated E_{10}) is a potent inhibitor of platelet

aggregation. The analogue E_{10} (diadenosine chloromethylene tetraphosphate) has the formula:

$$
\begin{array}{ccccccccccccccccccc}
 & & & & O & & & & O & & Cl & & O & & & & O & & \\
 & & & & \| & & & & \| & & | & & \| & & & & \| & & \\
\text{Adenosine} & - & O & - & P & - & O & - & P & - & C & - & P & - & O & - & P & - & O - \text{Adenosine} \\
 & & & & | & & & & | & & | & & | & & & & | & & \\
 & & & & O^- & & & & O^- & & H & & O^- & & & & O^- & &
\end{array}
$$

For purposes of the present invention, the term "AP_4A" includes the structure shown in Formula I and all functional equivalents thereof. An analog of AP_4A is AP_4A having a modification in one or more rings, in one or more of its substituents, or in both.

AP_4A has been shown to markedly inhibit ADP-induced platelet aggregation when it is administered to a mammal. Added before or during aggregation, AP_4A blunts the secondary wave response and causes rapid dispersion of aggregated platelets. The magnitude of inhibition has been shown to bear a direct relationship to the dose of AP_4A. Because platelet plugs form the bulk of arterial thrombi, a preferred therapeutic strategy to prevent thrombosis may be to utilize an agent (e.g., AP_4A, or an analog of AP_4A) that interferes with the adherence of platelets to vessel walls and to each other. Thus, in one embodiment of this invention, AP_4A, or one of its analogs (e.g. E_{10} or E_5), inhibits thrombus formation.

AP_4A has a short half-life in rabbit blood, both _in vivo_ and _ex vivo_ (platelets obtained from the blood of subjects who have received AP_4A). Compared to _in vivo_ clearance, the _ex vivo_ decay of

AP_4A is significantly longer. This may be explained by the use of citrated blood, which has been shown to inhibit the metal-ion dependent hydrolase responsible for the catabolism of AP_4A (Luthje, J. and Ogilvie, A., European Journal of Biochemistry, 149:119-127 (1985). This discovery is consistent with the previous observation that 90% of ^{32}P-labeled AP_4A added to normal plasma is degraded in 10 minutes when incubated at 37°C. Kim et al., Blood, 66:735-737 (1985). Endogenous platelet AP_4A, released in relatively high concentrations from the dense granules when stimulated platelets undergo the release phenomenon, may be important in modulating local platelet aggregation-dispersion. Thus, as described in the Example 1, an antithrombotic effect can be obtained by maintaining a high circulating AP_4A level. This observation suggests that AP_4A can be used as a clinical anti-platelet, antithrombotic agent.

AP_4A, E_{10} or other analogues may be used in the prevention of coronary and cerebrovascular thromboembolic events. Because platelet thrombi occur primarily in the arterial system, a preferred use of AP_4A or E_{10} is in the treatment of patients with a high risk of arterial thrombi in the heart and brain. In addition, AP_4A may be used in hemodialysis, in which patients are linked to artificial kidney machines, to prevent thrombosis in aterio-venous shunts. Furthermore, it is possible that AP_4A can be employed as a secondary prophylactic agent given to help prevent the recurrence of myocardial infarctions, strokes, and venous thrombosis

when present in an amount sufficient to inhibit platelet aggregation.

In general, AP_4A, or one of its analogs which inhibit platelet aggregation, can be administered intraperitoneally, intramuscularly, subcutaneously or via slow release encapsulation. However, the preferred method of administration is by intravenous injection. AP_4A can be introduced into the blood stream at any convenient point, although injection upstream from and near to the site of the suspected or known thrombus is preferred. An effective antithrombotic amount of AP_4A is that quantity which will prevent the formation of a thrombus. The actual quantity of AP_4A given in a specific case will vary according to the specific compound being utilized, the particular compositions formulated, the method of administration and the clinical needs of the patient. However, the dosage of this therapeutic agent generally is 0.01 to 10 mg/kg/day.

The therapeutic agent of the present invention, or a synthetic analog thereof, can be administered by injection in conjunction with a pharmacologically acceptable carrier, either alone or in combination with another drug (e.g., a thrombolytic agent). Acceptable pharmacological carriers are those which dissolve AP_4A or hold it in suspension, and which are compatible with physiological conditions. Examples of acceptable carriers are aqueous solutions of salts or non-ionic compounds such as sodium chloride or glucose, generally at an isotonic concentration. Other drugs may be present in the solution with AP_4A; it is important that such

additional components do not interfere with the ability of AP_4A to inhibit platelet aggregation. Those skilled in the art will know, or will be able to ascertain with no more than routine experimentation, particular pharmacological carriers for said composition.

The term drug is used in this description in its broadest sense and covers drugs useful to any mammal, including but not limited to, human beings, household animals and farm animals. The term drug is further used in describing this invention as including, but is not limited to, therapeutic drugs, diagnostic drugs and preventative drugs. A variety of classes, subclasses and specific examples of drugs not expressly mentioned herein are within the scope of this invention, and these other drugs will be well known or easily ascertainable to those skilled in the art.

In another embodiment of this invention, AP_4A, or one of its analogs, may inhibit a thrombus from growing by preventing the further aggregation of platelets at the periphery of the existing thrombus.

In yet another embodiment of this invention, AP_4A, or one of its analogs, such as E_{10}, which inhibit platelet aggregation, may assist also in the dissolution of existing thrombi or emboli when co-administered with a thrombolytic agent such as tissue plasminogen activator (TPA), streptokinase, or urokinase. For the purposes of this invention, the definition of co-administering includes (1) the simultaneous administration of AP_4A, or one of its analogs, and the thrombolytic agent and (2) the

administration of AP_4A or one of its analogs, shortly before or after the administration of the thrombolytic agent. Administration in this manner of AP_4A or one of its analogues will result in dispersion and/or prevention the reaggregation of platelets that are released from the blood clot in response to the action of the thrombolytic agent. Since AP_4A, or analogues thereof, act at a very early stage in thrombus formation, they are particularly useful when combined with clot-dissolving drugs currently available.

Ap_4A may be used in veterinary medicine. In such cases, AP_4A is preferably isolated from the same species of animal in which it is used, although cross-species use may be possible. In general, use in animals and humans is similar, although some variation in dosage requirements between species is expected.

The invention is illustrated further by the following examples, which are not to be taken as limiting in any way.

Example 1: Demonstration Of The Effects Of AP_4A On Blood Clotting

Methods and Materials

Animal Model of Arterial Thrombosis

In previous scientific reports, it was shown in a rabbit model that clot formation in an intra-carotid cannula can be modified by the administration of antiplatelet agents such as suloctidil,

aspirin and dipyridamole. Gurewich, V. and Lipinski, B. Thrombosis Research, 9:101 (1976); Louie, S. and Gurewich, V. Thrombosis Research, 30:323-335 (1983). The same model was used in demonstrating the antithrombotic activity of AP_4A.

Male, New Zealand white rabbits, weighing 2-2.5 kg., were anesthetized with ketamine hydrochloride (100 mg/kg intramuscularly). AP_4A (Boehringer Mannheim Biochemicals, Indianapolis, Indiana), or saline control was infused via a marginal ear vein. A segment of the left common carotid artery was isolated by vascular clamps. A 1 cm. length of polyethylene tubing (PE - 90, Clay Adams, Parsippany, NY) was inserted, secured by silk ligatures, and the blood flow re-established by removing the clamps. Blood was sampled from the right carotid artery for assays of AP_4A and ATP, and for platelet aggregation studies.

After preliminary trials, a standard AP_4A infusion protocol was established as follows: A dose of AP_4A at 50 mg/kg was reconstituted in 10 ml of normal saline and infused by pump at a uniform rate over two hours. Control rabbits received 10 ml of saline alone. The intracarotid cannula was inserted, and the re-establishment of blood flow timed at 15 minutes into the infusion. Upon the completion of infusion at 2 hours, the intracarotid tubing was removed, and its contents flushed out into a petri dish. The presence of a clot or of liquid blood contents was noted.

To avoid possible bias by minor changes in surgical technique, all the animal work was

performed by the same operator; rabbits were assigned to experimental or control groups at random.

Assay of Blood AP_4A and ATP

Blood samples were collected from the carotid artery through a catheter before and after (0, 10, 20, 40, and 60 minutes) infusion of AP_4A. Blood was anticoagulated by mixing with 0.15 volume of acid-citrate-dextrose solution. An aliquot of blood collected at the end of AP_4A infusion (t_o sample) was incubated at 37°C and sampled at 10, 20, 40, and 60 minutes to evaluate the *in vitro* decay of AP_4A. Blood samples of 115 ul each were admixed rapidly with 1,885 ul 3% perchloric acid and kept at 0°C for 30 minutes with intermittent vortexing. The acid soluble fraction was recovered by centrifugation at 1000 g for 10 minutes and neutralized by 5 M K_2CO_3. It was then kept at -80°C until assay of the nucleotides. The AP_4A assay was performed by coupling the phosphodiesterase and luciferase reactions in a luminometer (Model 6100 Picolite, Packard, Downers Grove, IL). The detailed method of AP_4A and ATP assays has been reported elsewhere (Kim, B. K., Chao, F. C., Leavitt, R., Fauci, A. S., Meyers, K. M. and Zamecnik, P. C. *Blood*, *66*:735-737, 1985).

Platelet Aggregation Studies

Rabbit carotid arterial blood was collected in 3.8% sodium citrate (9 volumes blood to 1 volume citrate). Platelet rich plasma (PRP) and platelet poor plasma (PPP) were prepared by centrifugation at

150 g and 1,000 g for 10 minutes respectively. Aggregation studies were performed in a Chrono-Log (Havertown, PA) aggregometer with ADP or collagen as aggregating agents. ADP (Sigma Chemical Co.) was used in a final concentration of 2 x 10^{-5}. Calf skin collagen (Sigma Chemical Co.) was used in a final concentration of 200 ug/ml.

Experimental Design and Statistical Analysis

Twenty-five rabbits each were assigned to the experimental group that received AP_4A (50 mg/kg), and the control group that received normal saline alone. The incidence of clot formation in the intracarotid cannula in the two groups was compared by the Chi-Square test.

Blood Levels of AP_4A and ATP

The disappearance of infused AP_4A in the circulation and in incubated blood was studied in 2 rabbits. Mean values of hemoglobin, hematocrit and platelet count were 10.1 g/dl, 30.8% and 362,000/ul respectively. The blood content of AP_4A in the rabbits was 51 nmol/l blood prior to infusion. This was 7.3 fold lower than the level observed in man, and comparable to the levels of AP_4A in the platelets of cats and cattle. Kim, B. K. et al., Blood, 66:735-737 (1985); Flodgaard, H., Zamecnik, P. C., Meyers, K. and Klenow, H., Thrombosis Research, 37:345-351 (1986). At the end of infusion it had increased to 125 fold of baseline (6.4 u mol/l blood). A very rapid disappearance of infused AP_4A was observed, with complete clearance within 10

minutes after infusion. When blood samples obtained at the end of AP_4A infusion were incubated at 37°C, only 15-fold and 4-fold levels of AP_4A, as compared to baseline could be detected after 10 minutes and 20 minutes respectively. The results indicated that the _ex vivo_ decay is slightly longer than the _in vivo_ clearance. On the other hand, the level of ATP showed bimodal increments: an initial increment and a late increment (Table 1).

The increased ATP level in the blood obtained at the end of AP_4A infusion may reflect an increase in plasma ATP, an immediate degradation product of AP_4A, plus an increase in blood cell ATP, generated from adenosine produced by AP_4A degradation during the 2 hours of infusion. A late increment of ATP at 60 minutes is most likely due to the result of increased intracellular ATP. These observations indicate that blood plasma contains a considerable amount of phosphomonoesterase as well as phosphodiesterase activity. The diminished response to ADP-induced aggregation seen in platelets recovered from AP_4A-infused rabbits was probably due to the combined effects of AP_4A and its degradation products such as ATP, AMP and adenosine.

The Effect of AP_4A on Platelet Aggregation

The effect of AP_4A on rabbit platelet aggregation by ADP and collagen was tested. Both ADP (2×10^{-5}M) and collagen (200 ug/ml) caused prompt and complete platelet aggregation, with a small primary wave and a sustained secondary wave of aggregation. Addition of AP_4A during aggregation blunted the

secondary wave response to ADP and caused the dispersion of aggregated platelets. The anti-aggregatory effect of AP_4A was detected at a

TABLE 1

Blood Contents of ATP and AP_4A in Rabbits: (Before and After Infusion of AP_4A)

		ATP, umol/l blood		AP_4A, umol/l blood	
		in vivo	ex vivo	in vivo	ex vivo
Before infusion		522.9	--	0.051	--
After infusion,	0 min	579.0	--	6.406	--
	10	573.5	570.1	0.043	0.799
	20	564.0	559.3	0.045	0.210
	40	562.0	551.5	0.050	0.041
	60	629.5	572.0	0.042	0.037

concentration of 2×10^{-4}M (tenfold that of ADP) and increased in a dose-response pattern with increasing concentrations (Figure 1). Similar results were obtained when AP_4A was added immediately before the initiation of aggregation by ADP (Figure 3A). However, AP_4A, while inhibiting slightly the collagen-induced aggregation when added prior to the induction of aggregation (Figure 3B), had no effect on the dispersion of preformed aggregates caused by collagen (Figure 2). Thus, the same dose of AP_4A

that causes almost complete inhibition of ADP-induced aggregation has little or no effect on collagen-induced platelet aggregation.

Figure 4 shows the results from platelet aggregation studies performed on blood from two rabbits receiving saline (Panel a) or AP_4A (Panel b) infusion. At an infusion dose of 50 mg per kg over 2 hours, AP_4A markedly blunted the aggregation of platelets induced by ADP (2×10^{-5}M), but had little effect on collagen-induced (200 ug/ml) aggregation.

The Effect of AP_4A Infusion on Thrombosis

Twenty-five rabbits received a constant infusion of AP_4A at a total dose of 50 mg/kg over 2 hours. Twenty-five control rabbits received saline infusion alone. The presence or absence of a clot in the intracarotid cannula was noted at the end of 2 hours. Of the 25 rabbits that received AP_4A, 14 were found to have formed clots in the intracarotid cannula, giving an incidence of thrombosis of 56%. Among the 25 saline controls, there were 21 clots, the incidence of thrombosis in the controls being 84% (p 0.05, Chi-Square test) (Table 2). The morphology of the intra-cannular thrombi has been described previously. Louie, S. and Gurewich, V. Thrombosis Research, 30:323-335 (1983). They consisted of a red body and a white head attached to the proximal or distal end of the cannula. Microscopically, large masses of platelets were separated by bands of fibrin, with other sections showing packed red cells and fibrin. There was no significant difference in dimension and weight between the

clots found in the AP_4A-infused rabbits and those recovered from the controls.

TABLE 2

TREATMENT	TOTAL RABBITS	CLOT PRESENT	CLOT ABSENT	% CLOTS	P
AP_4A	25	14	11	56	0.05
Saline	25	21	4	86	

Example 2: Demonstration Of The Effects Of Analogues of AP_4A On Blood Clotting

This Example illustrates that AP_4A analogues, especially the analogue designated as E_{10}, are potent inhibitors of platelet aggregation and blood clot formation.

Inhibition Of Platelet Aggregation By AP_4A Analogues

Human platelet-rich plasma was pre-incubated at 37°C with the appropriate analogue for 1 minute. Aggregation was then induced by 5 μM ADP. ID_{50} values (i.e. concentration of analogue at which platelets are inhibited by 50 percent) were obtained from log-dose response plots. Results showed that there is a wide-variation in inhibition of ADP-induced platelet aggregation among the analogues of AP_4A used (Table 3).

Table 3

Inhibitory Effects (ID50) Of Various Analogues Of Ap_4A on ADP-Induced Platelet Aggregation.

Analogue Designation	Agents	ID50, μM
E_1	$Ap(CH_2)pp(CH_2)pA$	>50
E_2	$App(CH_2)ppA$	22
E_3	$App(CH_2)_2ppA$	11
E_4	$Ap(CH_2)pp(CH_2)pA$	>50
E_5	App(CHF)ppA	4
E_6	Ap(CHF)pp(CHF)pA	50
E_7	$Ap(CF_2)pp(CF_2)pA$	15
E_8	$App(CF_2)ppA$	6
E_9	Ap(CHCl)pp(CHCl)pA	19
E_{10}	App(CHCl)ppA	3
E_{11}	$Ap(CCL_2)pp(CCl_2)pA$	9
E_{12}	$App(CCl_2)ppA$	10

Use of biphosponate analogues having P-C-P bridges located in the $P^2:P^3$ position resulted in greater inhibition than observed with other analogues. For example, the β-β-monochloromethylene derivative of AP_4A designated E_{10} (App(CHCl)ppA) was particularly effective, as was a monofluoro derivative, E_5 (App(CHP)ppA).

Figure 5 is a graphic representation of the data. Figure 5 shows aggregation of platelets in platelet-rich medium from human blood, in the presence of 5 μM ADP. The inhibitor analogues (12.5

μM) numbered 1-12 are cross-referenced to the analogues numbered in Table 3.

Under the conditions used, the β-β--monochloroethylene analogue of AP_4A (E_{10}) was the most effective inhibitor of platelet aggregation. The monofluoro analogue (E_5) was the next most effective in inhibiting platelet aggregation. Analogues E_1 and E_4 showed no effect on platelet inhibition, even at 50 μM.

The Effect Of E_{10} Infusion On Thrombosis

Initially, twelve rabbits received a constant infusion of E_{10} over a 2 hour period at a dosage of 100 mg in 10 ml. These intravenous infusions were performed as described above for AP_4A.

In addition, 30 mg of E_{10} in 3 ml saline was administered as a single injection over a one minute time span at the beginning of the cannulation period. Results are given in Table 4.

Table 4

Inhibitory Effects Of Diadenosine Chloromethylene Tetraphosphate On Intracarotid Artery Thrombosis

Treatment	Total No. Rabbits	Clot Present	Clot Absent	% Clots	P
E_{10} (100 mg)	12	4	8	33	0.05
(30 mg)	6	2	4	33	0.05
Total	18	6	12	33	0.025
Saline Control	15	12	3	80	---

As shown in Table 4, two-thirds of the injected rabbits (8 rabbits) showed no incidence of clotting. The Chi-square test shows this anti-thrombotic effect to be significant ($p < 0.05$). The E_{10} treatment at the 30 mg level shows a similar response but the sample size is too small to reveal a statistically significant effect of E_{10} on clot formation. When combined with the 100 mg series, the data show that E_{10} significantly reduces clot formation, as compared to saline controls.

Competitive Inhibition Of ADP-Induced Platelet Aggregation By E_{10}

Changes in light transmission reflecting the velocity of ADP-induced platelet aggregability was determined using a platelet aggregometer. Born, G.V.P., Nature 184:927-929 (1962). When the reciprocal of velocity (1/v) is plotted against the reciprocal of substrate (i.e. ADP) concentration, the inhibitory effects of AP_4A and E_{10} are revealed (Fig. 6). The kinetic plot is characteristic of competitive inhibition; in this double reciprocal plot only the slope is affected by the presence of inhibitor (AP_4A or E_{10}), the Y-intercepts remain constant. The Y points on the X-intercept are altered by factors $\left(1 + \frac{I}{K_i}\right)$ where I is the concentration of inhibitor, and K_i is a characteristic constant. Points on the X-intercept are given by the expression

$$\frac{-1}{K_m} \Big/ \left(1 + \frac{[I]}{K_i}\right) \quad \text{where} \quad \frac{-1}{K_m} \text{ is the}$$

intercept when [I] = 0. When [I] is known, the equation can be solved for K_i. In this Figure, the K_m for ADP is 3.0 μM, the K_i for AP_4A is 17.1 μM and 6.7 μM for E_{10}. This figure shows that E_{10} is superior to AP_4A as a competitive inhibitor of ADP-induced platelet aggregation.

EXAMPLE 3 The Effect of Sulfur-Containing AP_4A Analogues on Platelet Aggregation

This Example illustrates that sulfur-containing analogues of AP_4A are as effective as E_{10} in inhibiting aggregation of platelets. Platelet aggregation was induced by ADP and its inhibition measured as in the previous Example. Results show that sulfur-containing (i.e., those containing thiophosphate and thiophosphonate linkages) AP_4A analogues, E_{13}, E_{14} and E_{15}, have an inhibitory effect as great as E_{10} (Table 5).

Table 5

Effect of Sulfur-Containing AP_4A Analogues on ADP-Induced Blood Platelet Aggregation

Analogue Designation	Agents	ID50, μM
E_{10}	App(CHCl)ppA	<5
E_{13}	$Ap_sp(CHF)pp_sA$	7
E_{14}	$Ap_sp(CF_2)pp_sA$	17
E_{15}	Ap_sppp_sA	6

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

1. Use of diadenosine $5', 5'''-p^1, p^4$-tetraphosphate, or an analog thereof, for the manufacture of a medicament for inhibiting the formation of a thrombus in a mammal.

2. Use according to Claim 1, wherein the analog selected is from the group consisting of App(CHCl)ppA, App(CHF)ppA, App(CH_2)ppA, App(CHBr)ppA, Appp(CH_2)pA, Ap(CH_2)pp(CH_2)pA, (Sp,Sp)$Ap_spCH_2pp_sA$, $Ap_sp(CHF)pp_sA$, $Ap_sp(CF_2)pp_sA$, Ap_sppp_sA, (Rp,Rp)$Ap_spCH_2pp_sA$ and (Rp,Sp)$Ap_spCH_2pp_sA$.

3. Use of $5',5'''-p^1, p^4$-tetraphosphate, or an analog thereof, for the manufacture of a medicament for inhibiting coronary and cerebrovascular thromboembolic events in a mammal.

4. Use according to Claim 3, wherein the analog selected is from the group consisting of App(CHCl)ppA, App(CHF)ppA, App(CH_2)ppA, App(CHBr)ppA, Appp(CH_2)pA, Ap(CH_2)pp(CH_2)pA, (Sp,Sp)$Ap_spCH_2pp_sA$, $Ap_sp(CHF)pp_sA$, $Ap_sp(CF_2)pp_sA$, Ap_sppp_sA, (Rp,Rp)$Ap_spCH_2pp_sA$ and (Rp,Sp)$Ap_spCH_2pp_sA$.

5. A method for inhibiting the formation of a thrombus in a mammal, comprising administering to said mammal an effective antithrombic amount

of diadenosine 5', 5'''-p^1, p^4-tetraphosphate, or an analog thereof.

6. A method of Claim 1, wherein the analog selected is from the group consisting of App(CHCl)ppA, App(CHF)ppA, App(CH$_2$)ppA, App(CHBr)ppA, Appp(CH$_2$)pA, Ap(CH$_2$)pp(CH$_2$)pA, (Sp,Sp)Ap$_s$pCH$_2$pp$_s$A, Ap$_s$p(CHF)pp$_s$A, Ap$_s$p(CF$_2$)pp$_s$A, Ap$_s$ppp$_s$A, (Rp,Rp)Ap$_s$pCH$_2$pp$_s$A and (Rp,Sp)Ap$_s$pCH$_2$pp$_s$A.

7. In a composition for administration to a mammal for inhibiting the formation of a thrombus, the improvement comprising administering an effective antithrombotic amount of diadenosine 5', 5'''-p^1, p^4-tetraphosphate, or an analog thereof, and a pharmacologically acceptable carrier therefor.

8. A composition of Claim 7, wherein the analog selected is from the group consisting of App(CHCl)ppA, App(CHF)ppA, App(CH$_2$)ppA, App(CHBr)ppA, Appp(CH$_2$)pA, Ap(CH$_2$)pp(CH$_2$)pA, (Sp,Sp)Ap$_s$pCH$_2$pp$_s$A, Ap$_s$p(CHF)pp$_s$A, Ap$_s$p(CF$_2$)pp$_s$A, Ap$_s$ppp$_s$A, (Rp,Rp)Ap$_s$pCH$_2$pp$_s$A and (Rp,Sp)Ap$_s$pCH$_2$pp$_s$A.

9. Use of an effective thrombolytic amount of a thrombolytic agent in combintation with an effective antithrombotic amount of diadenosine 5',5'''-p^1,p^4-tetraphosphate, or an analog

thereof for the manufacture of a medicament for dissolving a thrombus in a mammal.

10. Use according to Claim 9, wherein the thrombolytic agent is selected from the group consisting of tissue plasminogen activator, streptokinase and urokinase.

11. Use according to Claim 10, wherein the analog selected is from the group consisting of App(CHCl)ppA, App(CHF)ppA, App(CH_2)ppA, App(CHBr)ppA, Appp(CH_2)pA, Ap(CH_2)pp(CH_2)pA, (Sp,Sp)$Ap_spCH_2pp_sA$, Ap_sp(CHF)pp_sA, $Ap_sp(CF_2)pp_sA$, Ap_sppp_sA, (Rp,Rp)$Ap_spCH_2pp_sA$ and (Rp,Sp)$Ap_spCH_2pp_sA$.

12. In a method for dissolving a thrombus in a mammal wherein a thrombolytic agent is administered to said mammal, the improvement comprising co-administering to said mammal an effective thrombolytic amount of a thrombolytic agent in conjunction with an effective antithrombotic amount of diadenosine 5',5'''-p^1,p^4-tetraphosphate, or an analog thereof.

13. A method of Claim 12, wherein the thrombolytic agent selected is from the group consisting of tissue plasminogen activator, streptokinase and urokinase and wherein the analog selected is from the group consisting of App(CHCl)ppA, App(CHF)ppA, App(CH_2)ppA, App(CHBr)ppA, Appp(CH_2)pA, Ap(CH_2)pp(CH_2)pA,

(Sp,Sp)$Ap_spCH_2pp_sA$, $Ap_sp(CHF)pp_sA$, $Ap_sp(CF_2)pp_sA$, Ap_sppp_sA, (Rp,Rp)$Ap_spCH_2pp_sA$ and (Rp,Sp)$Ap_spCH_2pp_sA$.

14. Use of 5',5'''-p^1,p^4-tetraphosphate, or an analog thereof for the manufacture of a medicament for inhibiting the growth of an existing thrombus in a mammal.

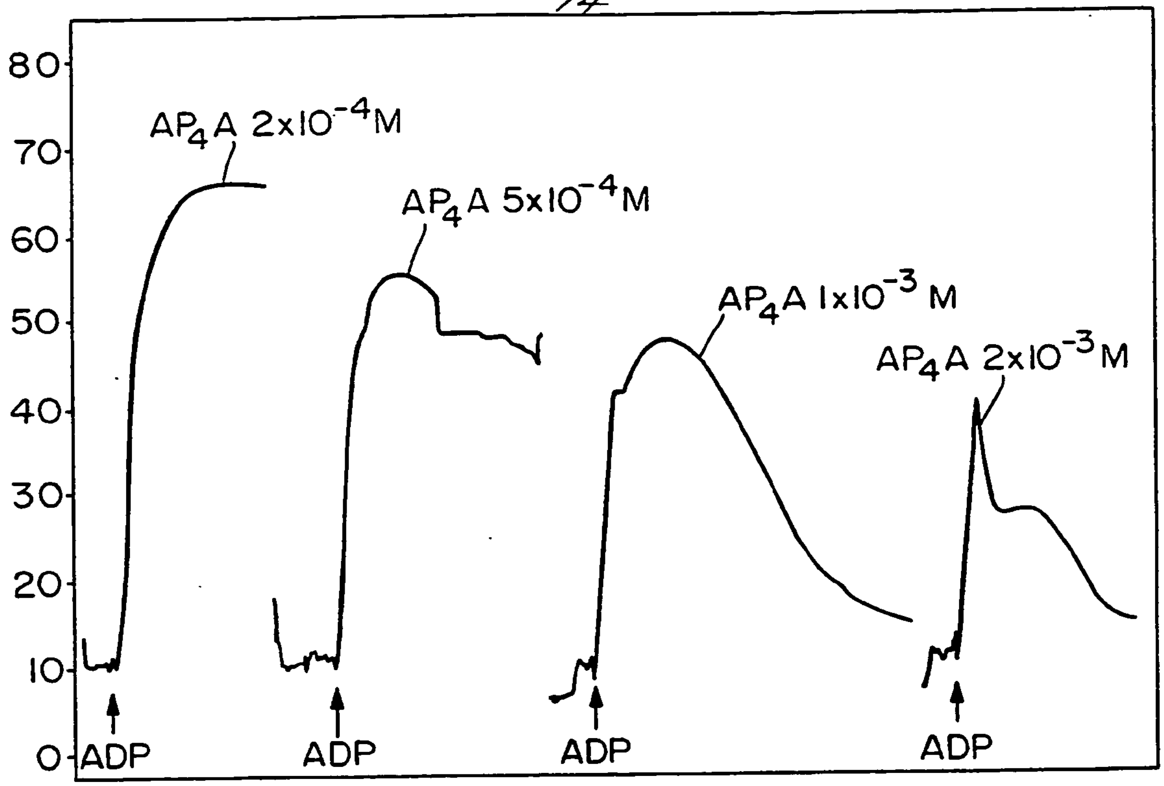
15. Use according to Claim 14, wherein the analog selected is from the group consisting of App(CHCl)ppA, App(CHF)ppA, App(CH_2)ppA, App(CHBr)ppA, Appp(CH_2)pA, Ap(CH_2)pp(CH_2)pA, (Sp,Sp)$Ap_spCH_2pp_sA$, $Ap_sp(CHF)pp_sA$, $Ap_sp(CF_2)pp_sA$, Ap_sppp_sA, (Rp,Rp)$Ap_spCH_2pp_sA$ and (Rp,Sp)$Ap_spCH_2pp_sA$.

16. A composition for administeration to a mammal for dissolving a thrombus, comprising an effective thrombolytic amount of a thrombolytic agent, an effective antithrombotic amount of diadenosine 5',5'''-p^1,p^4-tetraphosphate, or an analog thereof, and a pharmacologically acceptable carrier therefor.

17. The composition of Claim 16, wherein said thrombolytic agent is selected from the group consisting of tissue plasminogen activator, streptokinase and urokinase.

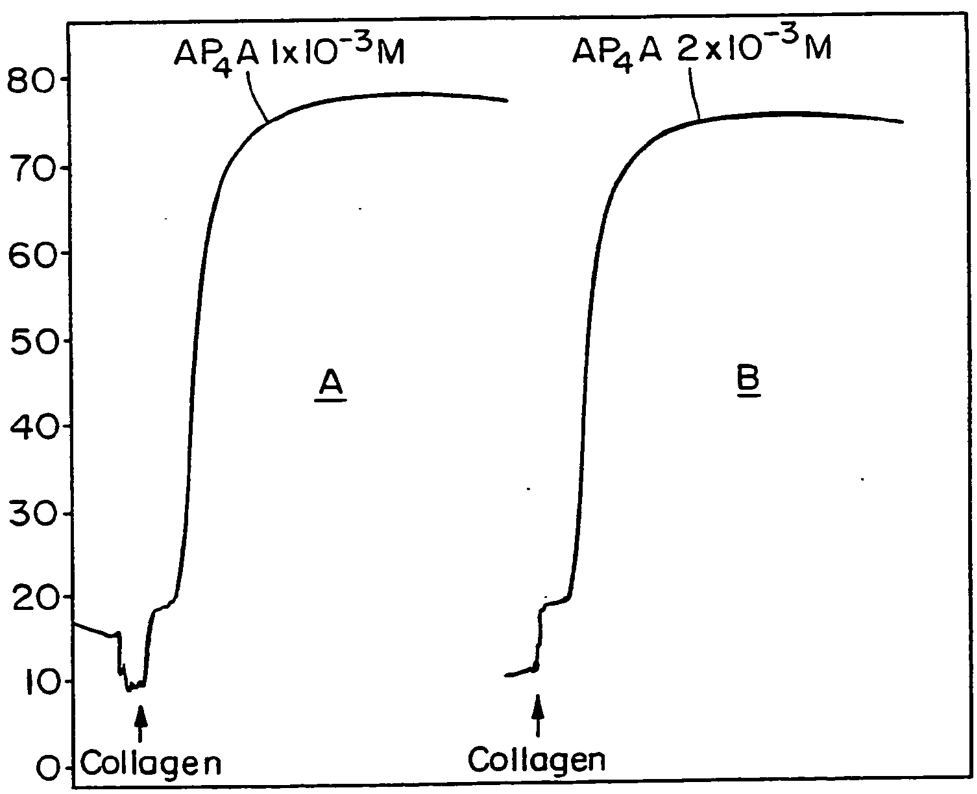
18. The composition of Claim 17, wherein the analog selected is from the group consisting of

App(CHCl)ppA, App(CHF)ppA, App(CH_2)ppA, App(CHBr)ppA, Appp(CH_2)pA, Ap(CH_2)pp(CH_2)pA, (Sp,Sp)Ap_spCH_2pp_sA, Ap_sp(CHF)pp_sA, Ap_sp(CF_2)pp_sA, Ap_sppp_sA, (Rp,Rp)Ap_spCH_2pp_sA and (Rp,Sp)Ap_spCH_2pp_sA.



80
70
60
50
40
30
20
10
0
AP4A 2x10-4M
AP4A 5x10-4M
AP4A 1x10-3 M
AP4A 2x10-3M
ADP
ADP
ADP
ADP

Fig. 1



80
70
60
50
40
30
20
10
0
AP4A 1x10-3M
AP4A 2x10-3M
A
B
Collagen
Collagen

Fig. 2

2/4

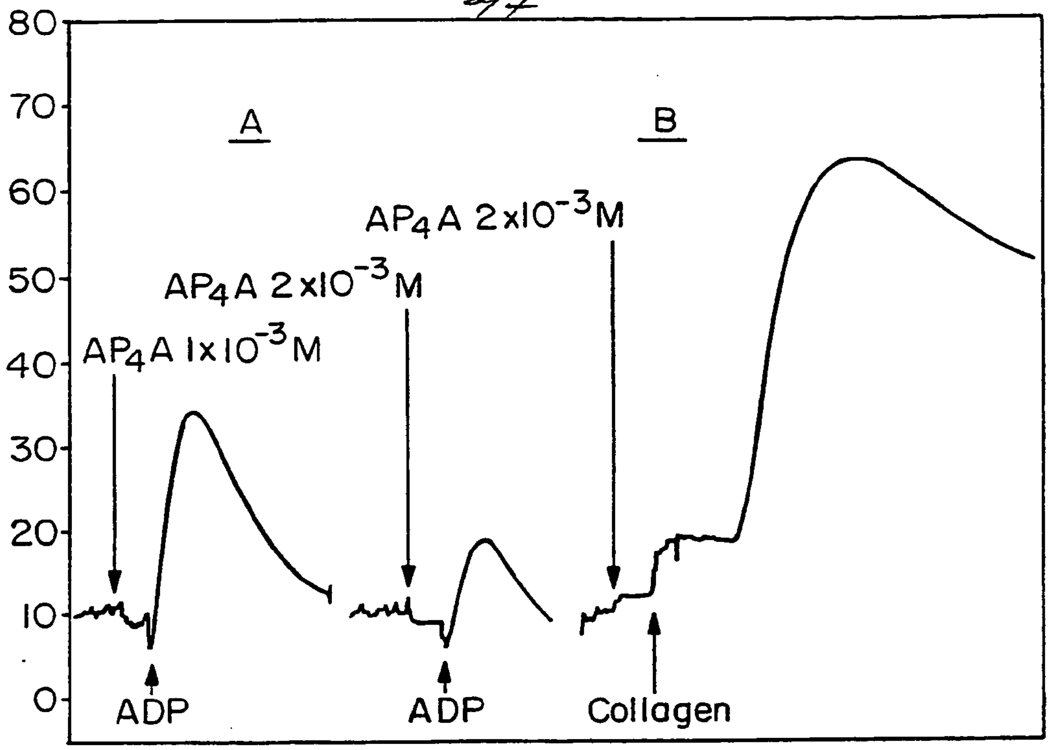
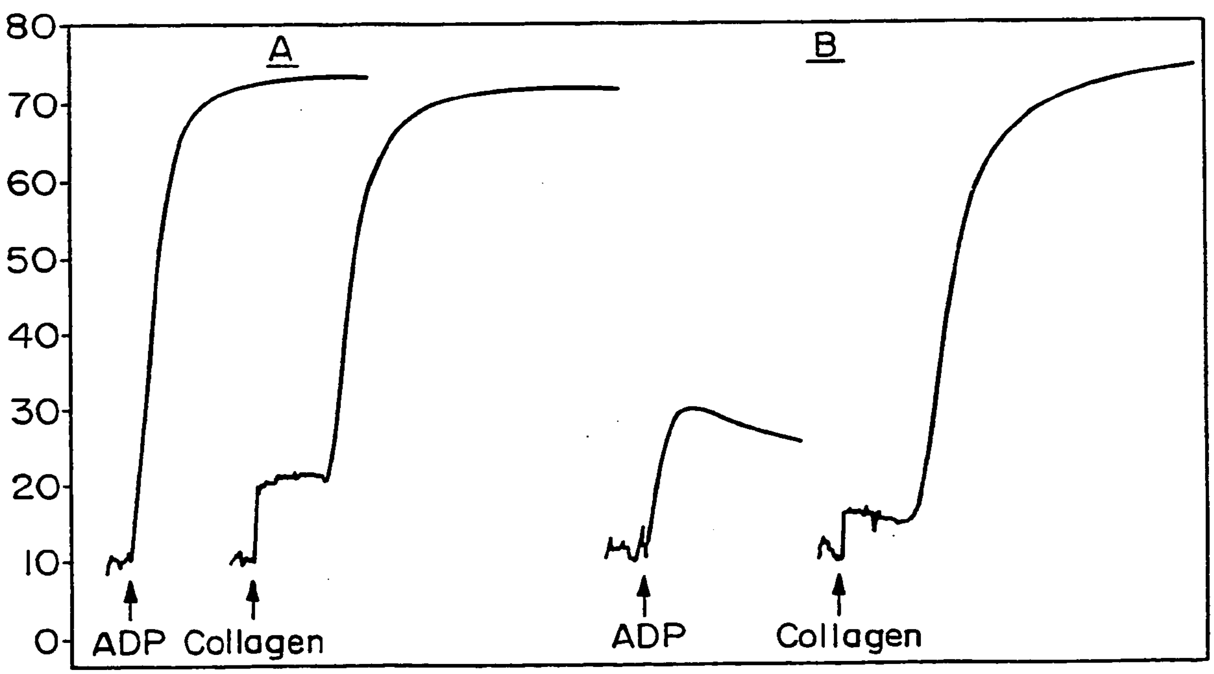

80
70
60
50
40
30
20
10
0
A
B
AP4A 1x10-3M
AP4A 2x10-3M
AP4A 2x10-3M
ADP
ADP
Collagen

Fig. 3


80
70
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20
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0
A
B
ADP
Collagen
ADP
Collagen

Fig. 4

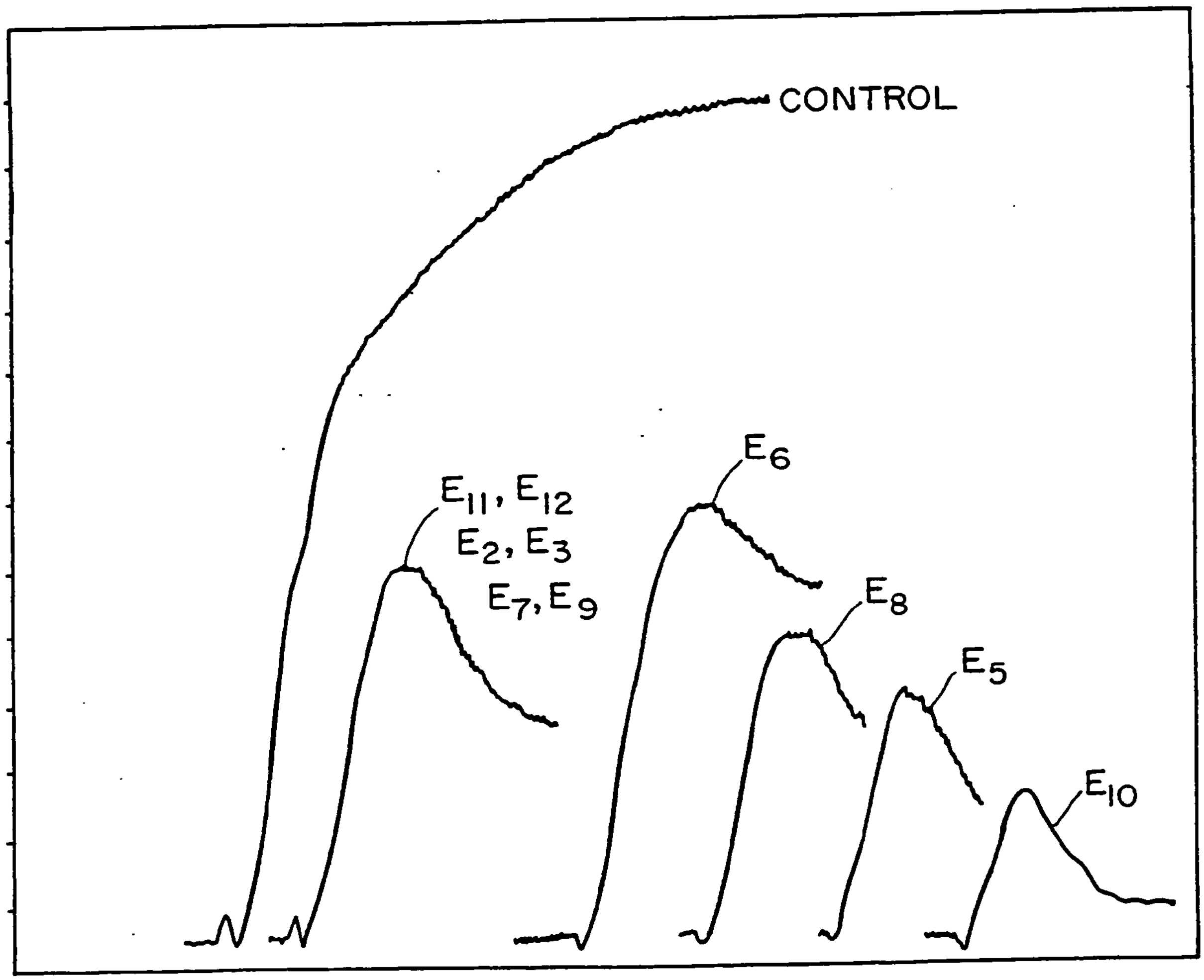

Fig. 5

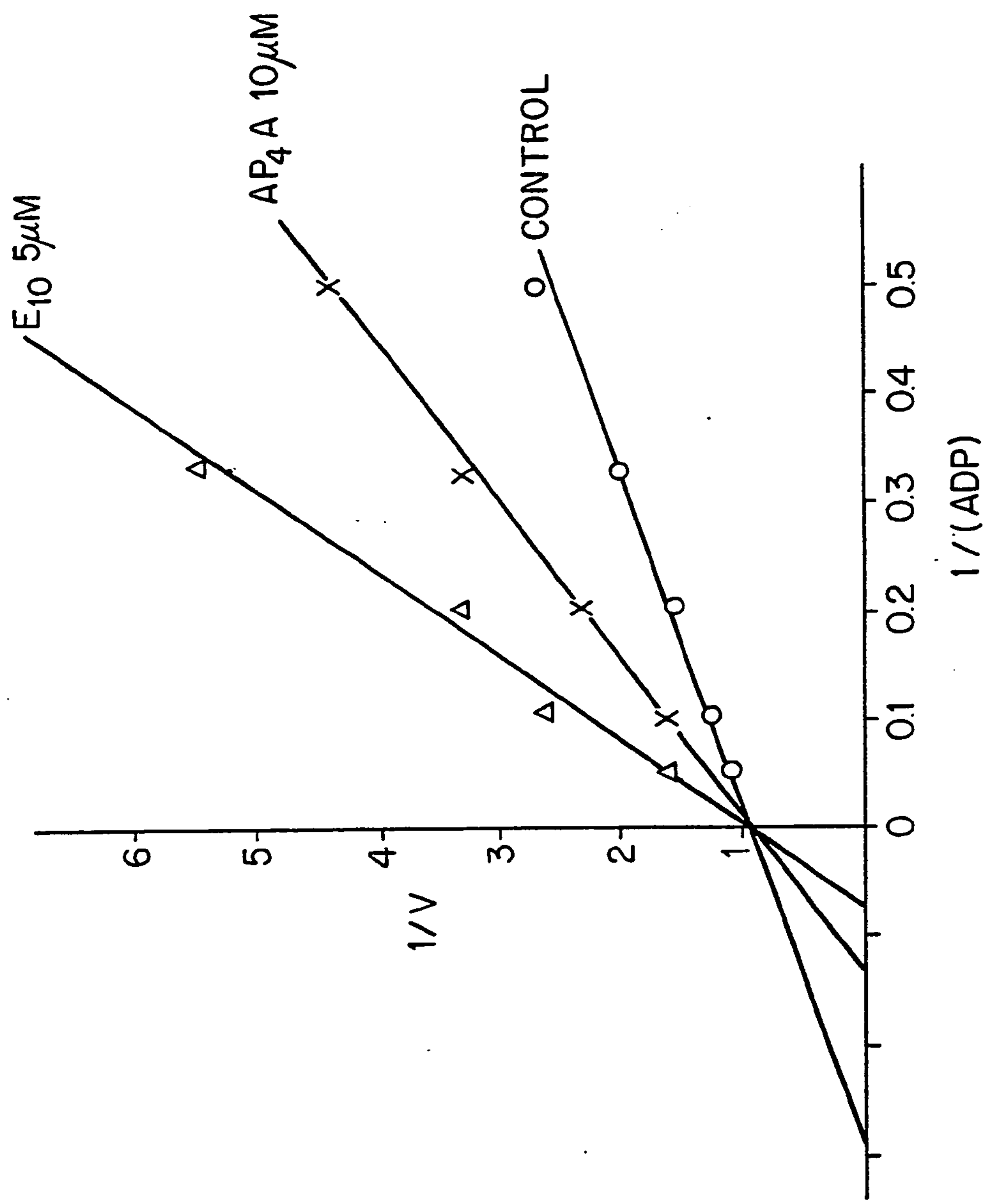

Fig. 6

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 88/03959

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC4: C 07 H 19/207, A 61 K 31/70

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC4	C 07 H; A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Hoppe-Seylers Zeitschrift für physiologische Chemie, Vol. 365, 1984 F.C. Chao et al.: "Inhibition of Platelet Aggregation by Ap_4A ", see page 610	1-4,14-15
Y	see the whole article	9-11, 16-18
	--	
X	Biochemical and biophysical research communications, Vol. 118, No. 3, 1984 J. Lüthje et al.: "Diadenosine triphosphate (Ap_3A) mediates human platelet aggregation by liberation of ADP ", see page 704 - page 709	1-4,14-15
Y	see the whole article	9-11, 16-18
	--	

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27th February 1989	15. 03. 89
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	The Merck Index, Vol. 10, 1983 (Rahway, N.J. USA) Martha Windholz et al.: "8683. Streptokinase" see page 1262 - page 1263 --	9-11,16-18
P,X	Chemical Abstracts, volume 109, no. 3, 18 July 1988, (Columbus, Ohio, US), Louie Stephen et al. : "Diadenosine 5',5'''-P^1,P^4-tetraphosphate, a potential antithrombotic agent. ", see page 33, abstract 16769n, & Thromb. Res. 1988, 49(6), 557- 65 --	1-4,14-15
P,X	EP, A2, 0 247 819 (UNITIKA LTD.) 2 December 1987, see particularly column 1 lines 11-26 --	1-4,14-15
P,X	Chemical Abstracts, volume 110, no. 6, 6 February 1989, (Columbus, Ohio, US), Nakajima Hiroshi et al. : "Prosthetic materials coated with antithrombogenic diadenosine tetraphosphate. ", see page 392, abstract 44985u, & Jpn. Kokai Tokkyo Koho JP 63,84,556 15 April 1988	1-4,14-15

International Application No. PCT/US88/03959

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 5-8,12-13/ because they relate to subject matter not required to be searched by this Authority, namely:

Method for treatment of the human or animal body by therapy, see PCT Rule 39.1(iv).

2. [X] Claim numbers 1,3,9,14 and 16/ because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The wordings "an analog thereof" and "a thrombolytic agent" are too broadly formulated to permit a meaningful search. The search on claims 1, 3, 9, 14 and 16 has therefore been incomplete.

3. [ ] Claim numbers.........., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/US 88/03959

SA 25586

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 12/01/89
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0 247 819	02/12/87	JP-A- 62278992	03/12/87

EPO FORM P0479

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82